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Decreased activity and impaired hormonal control of protein phosphatases in rat livers with a deficiency of phosphorylase kinase

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1. Livers from *gsd/gsd* rats, which do not express phosphorylase kinase activity, also contain much less particulate type-1 protein phosphatases. In comparison with normal Wistar rats, the glycogen/microsomal fraction contained 75% less glycogen-synthase phosphatase and 60% less phosphorylase phosphatase activity. This was largely due to a lower amount of the type-1 catalytic subunit in the particulate fraction. In the cytosol, the synthase phosphatase activity was also 50% lower, but the phosphorylase phosphatase activity was equal. 2. Both Wistar rats and *gsd/gsd* rats responded to an intravenous injection of insulin plus glucose with an acute increase (by 30–40%) in the phosphorylase phosphatase activity in the liver cytosol. In contrast, administration of glucagon or vasopressin provoked a rapid fall (by about 25%) in the cytosolic phosphorylase phosphatase activity in Wistar rats, but no change occurred in *gsd/gsd* rats. 3. Phosphorylase kinase was partially purified from liver and subsequently activated. Addition of a physiological amount of the activated enzyme to a liver cytosol from Wistar rats decreased the V of the phosphorylase phosphatase reaction by half, whereas the non-activated kinase had no effect. The kinase preparations did not change the activity of glycogen-synthase phosphatase, which does not respond to glucagon or vasopressin. Furthermore, the phosphorylase phosphatase activity was not affected by addition of physiological concentrations of homogeneous phosphorylase kinase from skeletal muscle (activated or non-activated). 4. It appears therefore that phosphorylase kinase plays an essential role in the transduction of the effect of glucagon and vasopressin to phosphorylase phosphatase. However, this inhibitory effect either is specific for the hepatic phosphorylase kinase, or is mediated by an unidentified protein that is a specific substrate of phosphorylase kinase.

INTRODUCTION

Type-1 (MgATP-dependent) and type-2A (polycation-stimulated) serine/threonine-protein phosphatases are the major enzymes involved in the dephosphorylation of phosphorylase and glycogen synthase [1,2]. The specific inhibition of type-1 phosphatases by phosphorylated inhibitor-1 and by the modulator protein (inhibitor-2) allows one to distinguish between the two groups. Thus, using purified hepatic synthase *b* as substrate, we found that the synthase phosphatase activity in the liver stems exclusively from type-1 protein phosphatases in the glycogen fraction and, to a lesser extent, in the cytosol [3]. In contrast, type-1 phosphatases in the glycogen fraction, the microsomal fraction and the cytosol, as well as cytosolic type-2A phosphatases, all contribute to the phosphorylase phosphatase activity in a postmitochondrial supernatant from liver [3,4].

Recent studies *in vivo* as well as on perfused livers showed that the phosphorylase phosphatase activity in a liver extract is decreased by 20–25% within 5–10 min after administration of glucagon and/or vasopressin [5,6]. The activity of synthase phosphatase was not affected [6]. On the other hand, after administration of insulin or of glucose, both the synthase phosphatase and phosphorylase phosphatase activities increased acutely by 20–30%, and these effects were additive. The effects of glucagon, insulin and glucose appear to be mediated by cytosolic transferable modifiers of the V of serine/threonine-protein phosphatases [6]. Previous work [5,6] argued against the involvement of inhibitor-1, which furthermore cannot be demonstrated in rat liver [7,8].

We have started to investigate in more detail the mechanism that underlies the acute inhibition of the phosphorylase phosphatase activity by glucagon and vasopressin. A well-known common target of these hormones is phosphorylase kinase. Glucagon increases the activity of the enzyme through phosphorylation by the cyclic-AMP-dependent protein kinase, and vasopressin stimulates phosphorylase kinase through an increased cytosolic concentration of Ca^{2+} [9]. The availability of *gsd/gsd* rats, which lack hepatic phosphorylase kinase activity [10], has allowed us now to establish the essential role of phosphorylase kinase in the acute inhibition of phosphorylase phosphatase by glucagon and vasopressin. An unexpected finding, however, was that livers from *gsd/gsd* rats contain rather low activities of phosphorylase phosphatase, and especially of synthase phosphatase.

EXPERIMENTAL

Materials and buffers

Calmodulin–Sepharose 4B was obtained from Pharm-

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acia LKB. Particulate liver glycogen was prepared by the phenol method [11]. The catalytic subunit of the cyclic-AMP-dependent protein kinase from bovine cardiac muscle [12] was given by Dr. D. A. Walsh (Davis, CA, U.S.A.). The standard buffer (buffer A) contained 50 mM-imidazole (pH 7.4), 0.5 mM-dithiothreitol, 4 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride and 0.5 mM-benzamidine. Throughout the purification of liver phosphorylase kinase, a solution with the following composition was used (buffer B): 50 mM-glycylglycine (pH 7.4), 0.3 mM-phenylmethanesulphonyl fluoride, 0.5 mM-benzamidine, 1 mM-dithiothreitol, 1 mM-2-mercaptoethanol and 10% (v/v) glycerol.

Handling of animals and livers

The treatment of the animals, the liver perfusions, the sampling and homogenization of the livers, the gel filtration and subcellular fractionation were essentially as described in ref. [6]. Concentrations of liver fractions are expressed with respect to the tissue from which they were derived; e.g., a 25% cytosol means the cytosol from 25 mg of liver in a final volume of 100 μl. Substances were injected intravenously at the following doses per kg body weight: 1 unit of insulin, 1 g of glucose, 0.4 unit of vasopressin, and 20 μg of glucagon. During liver perfusions insulin and glucose were added at initial concentrations of 50 m-units/ml and 50 mM respectively.

Purification and activation of phosphorylase kinase

The enzyme was purified to homogeneity from rabbit skeletal muscle [13]. For the partial purification of hepatic phosphorylase kinase the livers of 10 fed Wistar rats were homogenized in 1.5 vol. of buffer B plus 0.25 M-sucrose. The cytosolic fraction, prepared by high-speed centrifugation (35 min at 220000 *g*), was supplemented with solid $(NH_4)_2SO_4$ until 30% saturation and stirred for 1 h at 4 °C. The mixture was centrifuged (10 min at 15000 *g*) and the sedimented proteins were washed once in the original volume of buffer B with 30%-satd. $(NH_4)_2SO_4$. After re-sedimentation the pellet was dissolved in 50–100 ml of buffer B. Remaining salt was removed by overnight dialysis against buffer B. The solution was then cleared by centrifugation for 10 min at 10000 *g*, supplemented with 10 mM-$CaCl_2$ and applied to a calmodulin–Sepharose column (40 ml), equilibrated in buffer B plus 10 mM-$CaCl_2$. The retained kinase was eluted with buffer B containing 10 mM-EGTA and concentrated to about 5 ml in an Amicon ultrafiltration cell equipped with a PM30 membrane. This preparation was applied to a column of Sepharose 4B (500 ml) equilibrated in buffer B plus 50 mM-NaCl and 50 mM-NaF, and eluted with the same solution. The NaF was added in order to block irreversibly the contaminating protein phosphatase(s) [14]. The central peak of phosphorylase kinase, with the highest specific activity, was collected. After concentration to 1–2 ml by ultrafiltration as above, the preparation was dialysed overnight against buffer B without glycerol. At this stage, the specific activity of phosphorylase kinase was about 60 times that in the cytosol, with a recovery of 3% (Table 1). In one instance the state of activation of phosphorylase kinase was checked in the cytosolic fraction and found to be similar to that of the final enzyme preparation (result not shown).

Phosphorylation of the enzymes from muscle and liver was done by incubation for 60 min at 25 °C in a buffer containing 0.1 mM-$CaCl_2$, 125 mM-glycerol 2-phosphate (sodium salt) and 125 mM-Tris at pH 7.4, with (kinase *a*) or without (kinase *b*) 0.5 mM-ATP, 2.5 mM-magnesium acetate and 5 units (as defined in [12]) of the catalytic subunit of protein kinase/ml. Subsequently, the mixtures were extensively dialysed (20–30 h) against buffer B at 4 °C and stored at −20 °C in the presence of 60% glycerol.

Assays

Phosphorylase phosphatase was assayed with ^{32}P-labelled muscle phosphorylase *a*, and the synthase phosphatase activity was determined from the rate of activation of purified dog liver synthase *b*, as previously [6]. One unit of protein phosphatase converts 1 unit of substrate into product/min at 25 °C [6]. One unit of phosphorylase *a* and of synthase *a* incorporates 1 μmol of glucosyl units into glycogen under the conditions of the assays. The activity of phosphorylase kinase was derived from the rate of activation of purified phosphorylase *b*. Briefly, the preparation was incubated at 25 °C in the presence of 125 mM-glycerol 2-phosphate (sodium salt) and 125 mM-Tris at pH 7.4, 10 mg of muscle phosphorylase *b*/ml, 0.1 mM-$CaCl_2$, 50 mM-NaF, 3 mM-ATP and 5 mM-magnesium acetate. At regular time points for up to 10 min samples were taken for the determination of phosphorylase *a* at 25 °C [15]. One unit of phosphorylase kinase produces 1 unit of phosphorylase *a*/min. Since all the assays were performed at pH 7.4 instead of the classical pH 6.8, the maximally activated phosphorylase kinase from skeletal muscle was only about 3-fold more active than the non-activated enzyme [16,17]. Proteins were measured as described by Bradford [18], and glycogen was measured as described previously [15].

Table 1. Partial purification of phosphorylase kinase from rat liver

The data are the means of two preparations.

Fraction	Protein (mg)	Phosphorylase kinase		
		Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Cytosol	15375	0.12	100	1
$(NH_4)_2SO_4$ precipitate	392	2.3	48	19
Calmodulin–Sepharose	20	6.5	7	54
Sepharose 4B	8	7.0	3	58

Statistics

The results are means ± S.E.M. for the indicated number (n) of animals. Vertical bars in figures have the same meaning. Statistical differences for the data in Table 2 were calculated by Student's t test for independent random samples. The effects of hormone treatments were statistically analysed by Student's t test for paired data (absolute values) before and after treatment.

For kinetic analysis (Fig. 4) the phosphorylase phosphatase activity in each condition was expressed as a fraction of the maximal velocity in the control condition. The latter parameter was determined from a Lineweaver–Burk plot. Mean data from three experiments were then fitted to a Michaelis–Menten equation by non-linear regression analysis with the use of the 'Enzfitter' computer program (Elsevier-Biosoft). This program allows one to estimate the S.E.M. of the kinetic parameters.

RESULTS AND DISCUSSION

Activity of glycogen-synthase phosphatase in *gsd/gsd* livers

Watts & Malthus [19] observed that the time-dependent activation of the endogenous glycogen synthase occurred much more slowly during incubation of concentrated (25%) liver homogenates from *gsd/gsd* rats, in comparison with similar Wistar-liver preparations. Further experiments led those authors to the conclusion that the difference could be entirely explained by the higher concentration of glycogen in the *gsd/gsd*-liver homogenates. High glycogen concentrations are indeed known to inhibit glycogen-synthase phosphatase [19–21].

We have also compared the hepatic synthase phosphatase activity in Wistar rats and *gsd/gsd* rats, using a more quantitative assay. Since the total concentration of glycogen synthase in *gsd/gsd* livers is 50% higher than in Wistar rats [10], we used an excess of exogenous hepatic synthase *b*. On the other hand, only Wistar livers contain a significant concentration of phosphorylase *a* [10,15], which exerts a strong inhibitory effect on the glycogen-bound synthase phosphatase [22]. Therefore, AMP and Mg^{2+} were added to cancel the inhibition [21,22]. Since homogenates may contain various irrelevant inhibitors, we used liver extracts that had been desalted by filtration through Sephadex G-25. In those conditions, it became evident that the activity of glycogen-synthase phosphatase was about 75% lower in livers from *gsd/gsd* rats (Fig. 1). It is noteworthy that the difference was equally pronounced when concentrated (20%) liver extracts were used (Fig. 1*a*) and when the extracts were diluted an additional 10-fold (Fig. 1*b*). There still remained a 3-fold difference in synthase phosphatase activity when the glycogen concentration was artificially balanced in concentrated (20%) gel-filtered liver extracts by addition of α-particulate glycogen to the Wistar-liver extracts (results not shown). Thus the lower synthase phosphatase activity in *gsd/gsd*-liver extracts cannot be attributed to glycogen.

In general, the differences in protein phosphatase activities between Wistar and *gsd/gsd* rats were somewhat more pronounced when expressed per g of liver (result not shown) rather than per g of protein in the extract (Fig. 1, Table 2). This is due to the higher glycogen concentration in *gsd/gsd* livers, which results in a lower protein concentration.

Table 2 illustrates the subcellular distribution of the synthase phosphatase activity, when measured in minimally diluted fractions (25%). In *gsd/gsd* livers, as in normal Wistar livers [3,6], most of the activity was associated with the glycogen/microsomal fraction, which was therefore responsible for the large difference between Wistar and *gsd/gsd* noted in the postmitochondrial

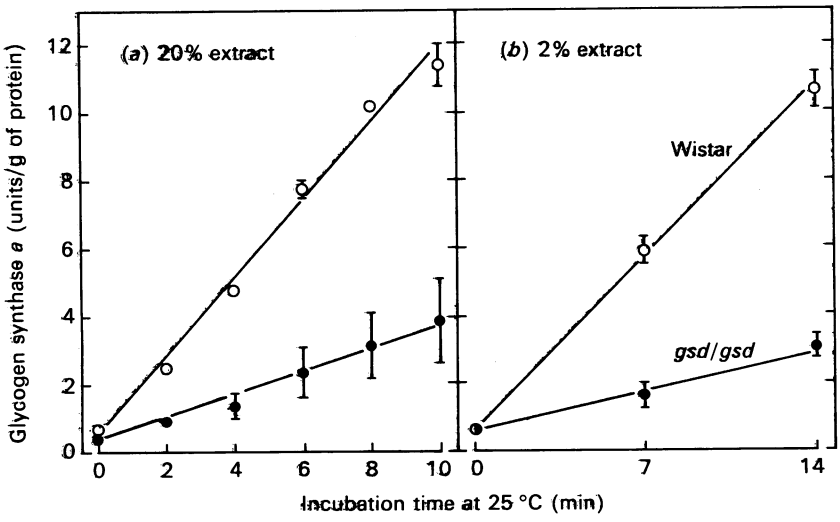

Fig. 1. Activation of glycogen synthase by liver extracts from Wistar rats or *gsd/gsd* rats

The postmitochondrial supernatant from the liver of three fed Wistar rats (○) and three fed *gsd/gsd* rats (●) was desalted on a Sephadex G-25 column and incubated at 25 °C in the presence of 3 mM-AMP, 5 mM-magnesium acetate and purified dog liver synthase *b* (1 unit/ml). The final concentration of the extracts was either 20% (*a*) or 2% (*b*). At the indicated times, samples were withdrawn for the assay of synthase *a*. The mean glycogen concentrations during the phosphatase assay on *gsd/gsd* and Wistar extracts were 29 and 15 mg/ml respectively in (*a*) and 7.5 and 6 mg/ml, respectively, in (*b*). These glycogen concentrations resulted from both the liver extracts and the added glycogen synthase.

Table 2. Glycogen-synthase phosphatase and phosphorylase phosphatase activities in liver fractions from Wistar and *gsd/gsd* rats

The synthase phosphatase and spontaneous phosphorylase phosphatase activities were measured in liver subcellular fractions at a final concentration of 25%. The phosphorylase phosphatase activity after trypsin treatment of the fractions was measured at a 5-fold higher dilution. The results are expressed in (m-)units/g of protein in the postmitochondrial supernatant, and represent means ± S.E.M. of 5–7 observations. The values in brackets indicate the fold stimulation by trypsin. [a]Significantly different from the corresponding value in Wistar rats ($P < 0.001$); [b]significantly different from the corresponding value in Wistar rats ($P < 0.05$); [c]not significantly different from the corresponding value in Wistar rats ($P > 0.40$).

	Synthase phosphatase (munits/g of protein		Phosphorylase phosphatase (units/g of protein)			
			Spontaneous		After trypsin	
Cell fraction	Wistar	*gsd/gsd*	Wistar	*gsd/gsd*	Wistar	*gsd/gsd*
Extract	1106±61	304±49[a]	72.4±5.4	34.5±29[a]	1617±63 [22]	1378±87[b] [40]
Cytosol	83±5	42±5[b]	30.4±2.7	33.3±2.0[c]	1194±69 [39]	1129±78[c] [34]
Glycogen/microsomes	840±125	190±47[a]	60.0±11.6	25.1±2.6[b]	964±151 [16]	577±19[b] [23]

supernatant. The cytosolic synthase phosphatase activity in *gsd/gsd* livers amounted to half of that measured in Wistar livers.

Phosphorylase phosphatase activity in *gsd/gsd* livers

In a postmitochondrial supernatant from *gsd/gsd* livers, the 'spontaneous' phosphorylase phosphatase activity was slightly less than half of that in the control group (Table 2). Upon subcellular fractionation it became evident that this difference stemmed solely from the glycogen/microsomal fraction, the cytosolic activities being strictly equal. At the rather high tissue concentrations used in these assays, the sum of the spontaneous phosphorylase phosphatase activities in the cytosol and in the glycogen/microsomal fraction was larger than that actually measured in the extract from which these fractions were prepared. This phenomenon has been noticed previously [6,23].

Preincubation of liver subcellular fractions with trypsin destroys the synthase phosphatase activity, but it increases the phosphorylase phosphatase activity manyfold ([3,22,23]; Table 2). This is due to the destruction of regulatory subunit(s) of type-1 phosphatases, resulting in the generation of free catalytic subunit. On the other hand, the activity of type-2 phosphatases is decreased by trypsin [24]. The trypsin-resistant phosphorylase phosphatase activities were still significantly lower in the postmitochondrial supernatant and in the glycogen/microsomal fraction from *gsd/gsd* rats (Table 2). However, the relative differences were smaller than for the corresponding 'spontaneous' phosphorylase phosphatase activities.

The above data indicate that Wistar and *gsd/gsd* livers contain comparable amounts of cytosolic type-1 and type-2A phosphorylase phosphatases. The lower glycogen/microsomal phosphorylase phosphatase activity in *gsd/gsd* rats appears to be due mostly to a lower concentration of particulate type-1 catalytic subunit. To a lesser extent, the difference is explained by an increased degree of inhibition of the particulate enzyme by regulatory subunits.

It remains to be seen why the particulate fraction of the *gsd/gsd* livers is markedly more deficient in synthase phosphatase than in phosphorylase phosphatase activity. The answer may lie in an intrinsic characteristic of the particulate phosphatases. In this respect it should be recalled that starvation of adrenalectomized rats results in an almost complete loss (> 90%) of the glycogen-bound synthase phosphatase activity, but only in a marginal fall (by 35%) in the glycogen-associated phosphorylase phosphatase activity [25]. However, it must also be borne in mind that the particulate synthase phosphatase activity belongs exclusively to the glycogen particles, whereas the phosphorylase phosphatase activity is about equally distributed among microsomes and glycogen [3]. An alternative explanation could therefore be that the microsomal phosphorylase phosphatase activities in Wistar and *gsd/gsd* rats are not significantly different.

The cause of the lower particulate type-1 phosphatase activities in *gsd/gsd* livers is not clear. The persistently high glycogen concentration could somehow provide a negative-feedback regulation of the concentration of the type-1 catalytic subunit or of the glycogen-anchoring subunit. Alternatively, it may be that phosphorylase kinase itself is required for the full expression of particulate type-1 protein phosphatases, or that phosphorylase kinase and the latter phosphatases have a common subunit.

Acute hormonal regulation of the phosphorylase phosphatase activity

For several reasons, the hormonal effects were specifically assessed on the phosphorylase phosphatase activity in the cytosolic fraction. First, no differences were detected between the cytosolic phosphorylase phosphatase activities in Wistar and *gsd/gsd* livers (Table 2). Second, the hormone effects appear to require cytosolic mediators, and they are fully expressed on the cytosolic protein phosphatases [6]. Finally, glucagon and vasopressin inhibit phosphorylase phosphatase, but not glycogen-synthase phosphatase [6].

Fig. 2 shows that an intravenous injection of insulin plus glucose increased the cytosolic phosphorylase phosphatase activity in both groups, although the response was slightly smaller in *gsd/gsd* livers. Similar results (not shown) were obtained with perfused livers, where the cytosolic phosphorylase phosphatase activity 10 min after the addition of insulin plus glucose had increased by 35±6% in Wistar rats ($n = 6$; $P = 0.007$) and by 26±10% in *gsd/gsd* rats ($n = 5$; $P = 0.03$). This shows that phosphorylase kinase is not required for the trans-

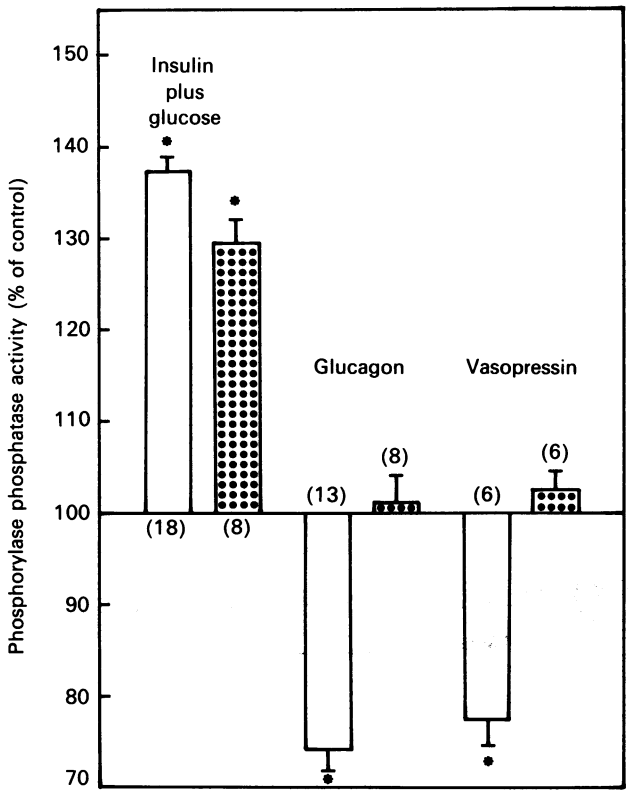

Fig. 2. Effect of an injection of insulin plus glucose, of vasopressin or of glucagon on the phosphorylase phosphatase activity in liver cytosol

The phosphorylase phosphatase activity in the cytosol of a liver sample taken 5 min after the indicated intravenous injection is expressed as a percentage of the activity in a liver sample obtained immediately before treatment, for the indicated numbers of Wistar (□) and *gsd/gsd* (▦) rats: *significant difference from the pre-treatment value ($P < 0.01$).

duction of the insulin and glucose signals to phosphorylase phosphatase. It leaves open the possibility that the inactivation of phosphorylase kinase after injection of insulin [5,26] is the result of an increased phosphorylase-kinase phosphatase activity.

We have shown previously that the addition of glucagon and/or vasopressin to perfused Wistar livers results in an acute 25% fall in the cytosolic phosphorylase phosphatase activity [6]. Fig. 2 confirms that either hormone provokes this effect within 5 min after intravenous injection into Wistar rats. However, the same treatments were entirely ineffective in *gsd/gsd* rats. These observations suggest that the effects of glucagon and vasopressin on phosphorylase phosphatase are mediated by their actions on phosphorylase kinase.

Control of phosphorylase phosphatase by phosphorylase kinase

Earlier work indicated that the acute effect of glucagon on the hepatic phosphorylase phosphatase activity is mediated by a transferable cytosolic inhibitor that does not require MgATP *in vitro* [6]. Therefore, if phosphorylase kinase is this putative mediator, it cannot act by phosphorylating phosphorylase phosphatase. One possibility is another physical interaction between kinase and phosphatase; that interaction should then be triggered or enhanced by a glucagon-induced covalent modification of phosphorylase kinase. Alternatively, the glucagon-activated phosphorylase kinase could phosphorylate *in vivo* an unidentified cytosolic protein which then inhibits phosphorylase phosphatase. The latter hypothesis would also easily explain how vasopressin shares the effect of glucagon on phosphorylase phosphatase [6] without causing a stable activation of phosphorylase kinase [27,28].

Basic observations. We have started to test these hypotheses by investigating the effect of added purified phosphorylase kinase on the cytosolic phosphorylase phosphatase activity. The concentration of phosphorylase kinase in liver is much lower than in muscle; up to now only one group has succeeded in obtaining analytical quantities of the homogeneous enzyme, from the glycogen fraction of 1000 rat livers [29]. We have aimed at a partial, but well reproducible, purification of the much more abundant cytosolic phosphorylase kinase. Fig. 3(*a*) shows that the preparation contained predominantly non-activated phosphorylase kinase (*b* form), since the activity was increased 4-fold by incubation with the C-subunit of cyclic-AMP-dependent protein kinase, MgATP and Ca^{2+} (*a*-form). Neither preparation of phosphorylase kinase contained detectable phosphorylase phosphatase activity (Fig. 3*b*). Fig. 3(*b*) also shows that the activity of phosphorylase phosphatase in a minimally diluted liver cytosol was not appreciably affected by addition of partially purified liver phosphorylase kinase *b*. The amount of added phosphorylase kinase was roughly equal to that already present in the 20% cytosol. (Fig. 3*a*). However, when the same amount of liver phosphorylase kinase was activated before addition to the cytosol, the preparation decreased the phosphorylase phosphatase activity by half (Fig. 3*b*).

Activation of phosphorylase kinase in the presence of 1 mM-EGTA instead of 0.1 mM-$CaCl_2$ did not appreciably affect the final extent of activation. Also, the resulting activated phosphorylase kinase caused an equally large inhibition of phosphorylase phosphatase (result not shown). This argues against a requirement for Ca^{2+}-dependent proteolysis in the activation of the kinase.

Requirement for activated phosphorylase kinase. ATP is a strong inhibitor of protein phosphatases [30–32]. It can be excluded, however, that the data in Fig. 3(*b*) reflect inhibition by ATP. Indeed, the concentration of ATP that was transferred from the dialysed preparations of phosphorylase kinase to the phosphatase assay mixture was below 1 μM, and even 10 μM-ATP did not inhibit the cytosolic phosphorylase phosphatase (results not shown). Further, when bovine serum albumin (5 mg/ml) was incubated and dialysed exactly as was done with phosphorylase kinase, it did not affect the phosphatase activity (result not shown).

Since our preparations of liver phosphorylase kinase are impure, it appears important to establish that the inhibition of phosphorylase phosphatase is indeed caused (directly or indirectly) by activated phosphorylase kinase. Accordingly we have subjected the cytosol from ten *gsd/gsd* livers to the phosphorylase kinase purification procedure. The final preparation was tested as in Fig. 3 before as well as after incubation in 'activation' condi-

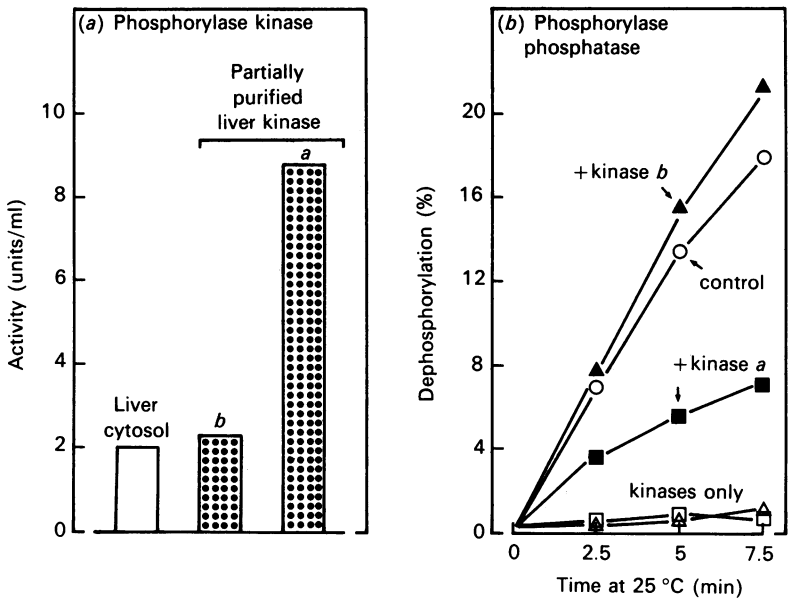

Fig. 3. Effect of partially purified liver phosphorylase kinase on the cytosolic phosphorylase phosphatase activity from Wistar rat liver

(*a*) Activity of phosphorylase kinase in a 20% liver cytosol and in partially purified preparations of phosphorylase kinase *a* and *b*. (*b*) Time-dependent dephosphorylation of phosphorylase *a* by phosphorylase phosphatase present in the liver cytosol (control), in the preparations of phosphorylase kinase *b* (△) and *a* (□), and in mixtures of cytosol and kinase preparations.

Table 3. Effect of partially purified liver phosphorylase kinase on the activities of phosphorylase phosphatase and glycogen-synthase phosphatase in Wistar and *gsd/gsd* livers

The protein phosphatase activities were measured in liver subcellular fractions at a final concentration of 10–20%. Results are expressed as means ± S.E.M. for *n* observations [a]No significant difference between the effect of the two phosphorylase kinase preparations ($P \geqslant 0.27$); [b]significant difference between the effect of the two phosphorylase kinase preparations ($P \leqslant 0.016$).

Rat strain	Cell fraction (*n*)	Addition	Activity (% of control) Synthase phosphatase	Phosphorylase phosphatase
Wistar	Cytosol (5)	None	100	100
		Kinase *b*	100 ± 1	110 ± 9
		Kinase *a*	100 ± 1[a]	57 ± 6[b]
	Glycogen/ microsomes (4)	None	100	100
		Kinase *b*	95 ± 3	101 ± 5
		Kinase *a*	96 ± 3[a]	60 ± 7[b]
gsd/gsd	Cytosol (3)	None	–	100
		Kinase *b*	–	110 ± 2
		Kinase *a*	–	60 ± 2[b]

tions. Either preparation failed to affect the cytosolic phosphorylase phosphatase activity (results not shown).

The free regulatory subunits of the type-I [33] and type-II [34] cyclic-AMP-dependent protein kinases inhibit several protein phosphatases that act on phosphorylase *a*. Therefore we used free C-subunit in the activation of phosphorylase kinase. Also, since the preparations of non-activated phosphorylase kinase were not inhibitory (Fig. 3*b*, Table 2), the contribution of endogenous R-subunit must be negligible. However, it has been shown that the inhibitory effect of the type-II R-subunit increases considerably upon phosphorylation or thiophosphorylation [35,36]. Two lines of evidence seem to exclude that free phosphorylated R-subunit is the actual inhibitor in the preparations of activated phosphorylase kinase. First, the inhibition requires dissociated R-subunit [36]. Yet addition of an excess of C-subunit to a preparation of activated phosphorylase kinase did not interfere with the inhibition of phosphorylase phosphatase (results not shown). Second, it has recently been described that the phosphorylated R-subunit is a purely competitive inhibitor with respect to phosphorylase *a* [37]. This contrasts with the non-competitive character of the inhibition that we observe (see below).

Characteristics of the inhibition. To date we have examined six preparations of liver phosphorylase kinase that behaved similarly (Table 3). A few preparations were also added to the cytosol prepared from *gsd/gsd*

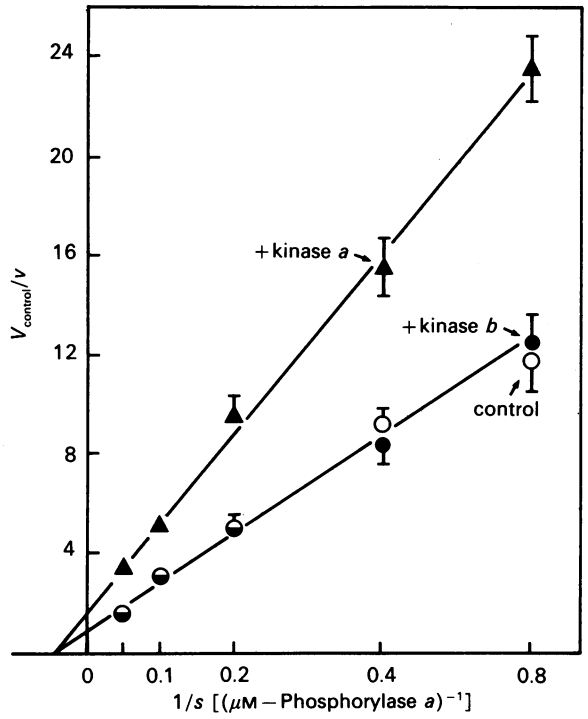

Fig. 4. Effect of partially purified liver phosphorylase kinase on the substrate-saturation kinetics of the cytosolic phosphorylase phosphatase

The experiments were performed as described in the legend to Fig. 3, except for the determination of the phosphorylase phosphatase activities at various concentrations of phosphorylase *a* (expressed as monomer). The data from three experiments were each computed as a fraction of the corresponding control *V* and analysed as described under 'Statistics'. The computer-generated K_m values were 27.7±4.1 μM (○), 23.1±1.2 μM (●) and 30.6±3.2 μM (▲). The corresponding *V* values (in units of phosphorylase *a* inactivated/min per ml of assay mixture) were 1.02±0.1 (○), 0.93±0.03 (●) and 0.64±0.04 (▲).

rats (Table 3); in this instance phosphorylase phosphatase was also inhibited exclusively by the activated kinase preparation, and to the same extent. This indicates that the *gsd/gsd* livers contain the basic elements for a response to glucagon, except for a protein that is present in our preparations of liver phosphorylase kinase. Table 3 also shows that the preparations of phosphorylase kinase *a* exerted the same specific inhibition on the phosphorylase phosphatase activity associated with the particulate fraction (glycogen particles plus microsomal fraction). This is in agreement with our previous conclusion [6] that glucagon inhibits the particulate phosphorylase phosphatase through a cytoplasmic mediator.

In previous studies on perfused livers, neither glucagon nor vasopressin affected the activity of glycogen-synthase phosphatase [6]. In keeping with those observations, the preparations of liver phosphorylase kinase failed to affect the activity of glycogen-synthase phosphatase, whether cytosolic or particulate (Table 3).

Kinetic mechanism of the inhibition. Phosphorylated phosphorylase kinase could obviously compete with phosphorylase *a* for the same cytosolic protein phosphatase [4]. However, Fig. 4 illustrates that partially purified phosphorylase kinase *a* from liver affected specifically the *V* of the cytosolic phosphorylase phosphatase. In addition, the effect of activated phosphorylase kinase has also been measured on the phosphorylase phosphatase activity in the glycogen/microsomes fraction (cf. Table 3), but at two widely different concentrations of phosphorylase *a* (1.25 μM and 30 μM; cf. Fig. 4). The residual phosphorylase phosphatase activities, with respect to the control, were 69±8% and 68±4% respectively ($n = 3$). This indicates a non-competitive inhibition. It is important to note that the same kinetic mechanism accounts for the acute inhibition of phosphorylase phosphatase by glucagon in the perfused liver [6].

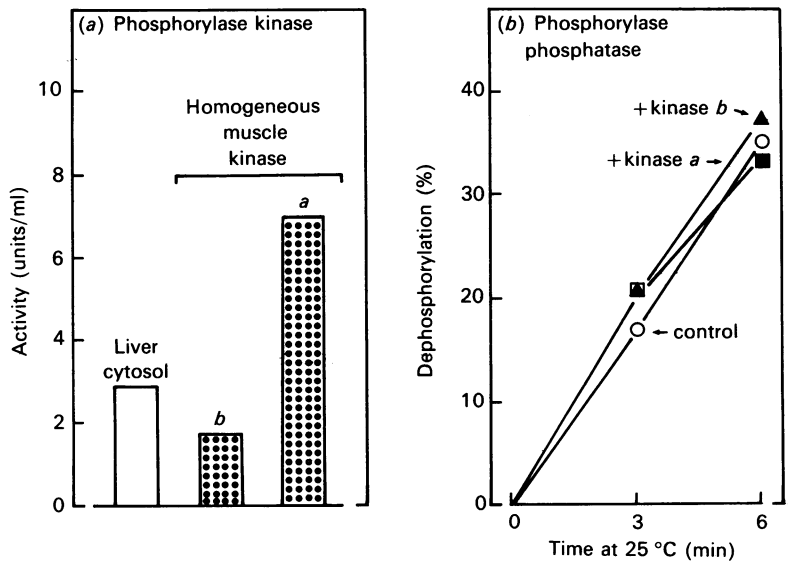

Fig. 5. Effect of homogeneous muscle phosphorylase kinase on the cytosolic phosphorylase phosphatase activity from Wistar-rat liver

General procedure and symbols as in the legend to Fig. 3, except for the absence of phosphorylase phosphatase activity from the purified kinases, which is not illustrated. The apparently modest increase in the phosphorylase kinase activity upon activation is due to the assay of the kinase at pH 7.4 (see under 'Assays').

Direct or indirect inhibition by activated phosphorylase kinase? Although the above results provide compelling evidence for the role of phosphorylase kinase *a* in the signal transduction from the glucagon receptor to phosphorylase phosphatase, they do not prove that it is the activated phosphorylase kinase that interacts directly with the phosphatase. In an effort to solve this problem, experiments similar to that shown in Fig. 3 were also carried out with homogeneously pure phosphorylase kinase from skeletal muscle. However, the cytosolic phosphorylase phosphatase activity was not affected by addition of the latter enzyme (either non-activated or maximally activated), at 'physiological' concentrations (Fig. 5) or even at a 10-fold higher concentration (results not shown). Unfortunately, the latter results do not allow us to decide whether the inhibitory action on the phosphorylase phosphatase activity is specific for the active phosphorylase kinase from liver, or whether the actual inhibitor is a co-purifying specific substrate of liver phosphorylase kinase. The elucidation of the latter question will require the purification to homogeneity of the inhibitory phosphoprotein.

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